Inhibition of Human and Simian Immunodeficiency Virus Protease Function by Targeting Vpx-Protease-Mutant Fusion Protein into Viral Particles

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The human immunodeficiency virus type 1 (HIV-1) Vpr and HIV-2 Vpx proteins package into virions through interactions with their cognate Gag polyprotein precursor. The targeting properties of Vpr and Vpx have been exploited to incorporate foreign proteins into virions by expression as heterologous fusion molecules (X. Wu, H.-M. Liu, H. Xiao, J. Kim, P. Seshiah, G. Natsoulis, J. D. Boeke, B. H. Hahn, and J. C. Kappes, *J. Virol.* 69:3389–3398, 1995). To explore the possibility of utilizing Vpx and Vpr to target dominant negative mutants of the HIV Pol proteins into virions, we fused HIV-2 Vpx with an enzymatically defective protease (PR) mutant. Using a vector system to facilitate transient coexpression with HIV provirus, Vpx-PR-mutant (VpxPR^M) fusion protein was expressed and packaged efficiently into HIV-2 and simian immunodeficiency virus virions. Immunoblot analysis of purified virions demonstrated that the packaging of VpxPR^M interfered with the processing of the Gag and Gag/Pol precursor proteins, similar to that of a well-characterized active-site PR inhibitor. The incomplete processing of Gag and Gag/Pol was consistent with a 25-fold reduction in virion infectivity. The coexpression of a packaging defective VpxPR^M fusion protein with HIV-2 provirus produced virions with fully processed Gag protein, similar to wild-type virions. Importantly, virions *trans* complemented with a Vpx-chloramphenicol acetyltransferase fusion protein were normal with respect to the processing of Gag protein and the ability to infect and replicate *in vitro*. These results indicate that VpxPR^M specifically inhibited the function of the viral protease and provide for the first time proof of principle that the incorporation of foreign proteins into virions via fusion with Vpx can inhibit HIV replication. The use of accessory proteins as vehicles to deliver deleterious proteins to virions, including dominant negative mutants of Pol proteins, may provide new opportunities for application of gene therapy-based antiretroviral strategies. The ability to package PR by expression *in trans*, independent of the Gag/Pol precursor, also represents a novel approach that may be exploited to study the function of the Pol proteins.

Human and simian immunodeficiency virus (HIV and SIV) Gag and Pol proteins are synthesized as precursor polyproteins that are cleaved into mature products by the viral protease (7, 25, 28, 44). The Gag precursor protein (Pr55) is processed to yield matrix (MA), capsid (CA), nucleocapsid (NC), and p6, while the Pol precursor is processed into protease (PR), reverse transcriptase (RT), and integrase (IN). PR, RT, and IN are synthesized and assembled into virions in the context of the Gag/Pol polyprotein, which is expressed by a ribosomal frameshift at the level of approximately 5 to 10% that of Gag protein (19). PR function is essential for maturation of viral core proteins and the generation of infectious virions (25, 34, 44). Although fully processed forms of Gag can be detected within the cytoplasm of T lymphocytes lytically infected with HIV type 1 (HIV-1), activation of PR function is limited until assembly and release of the viral particle (20, 36). The functional HIV protease is a homodimeric molecular complex. Each monomer contributes one of the two catalytic Asp-Thr-Gly active-site triads. Mutagenesis of the active site has revealed that substitution of the conserved aspartic acid residue at position 25 is sufficient to inactivate the enzyme and to completely inhibit HIV-1 replication (25, 34). Mutational studies on intramolecular PR dimers (two PR coding regions genetically linked to form a dimeric gene) indicate that mutation of

even a single aspartic acid is sufficient to abolish enzyme activity of the complex when expressed in *Escherichia coli* (9).

In addition to Gag, Pol, and Env, HIV and SIV virions have been shown to contain viral accessory proteins, including Vpx (18, 23), Vpr (4, 56, 57), and Vif (33). Vif is present in all primate lentiviruses, all but SIV_{AGM} contain Vpr, and Vpx is unique to the HIV-2/SIV_{SM}/SIV group of viruses (49, 52). Vpx and Vpr are capable of packaging into virions in amounts comparable to that of Gag. Vif, on the other hand, has been detected in amounts similar to those of Pol proteins (33). Vpx, Vpr, and Vif are important for efficient virus replication of HIV and SIV in natural target cells (5, 13, 15, 17, 22, 40, 50, 51). HIV-1 Vpr and HIV-2 Vpx proteins are known to be packaged by interactions with the p6 portion of their cognate Gag polyprotein precursors (24, 26, 31, 35, 42, 54). Because Vpx and Vpr are not required for virus replication in immortalized T-cell lines and can be packaged efficiently by expression *in trans*, we tested whether they could be exploited to target foreign protein to HIV and SIV virions. Those studies demonstrated efficient packaging of enzymatically active Vpr-Vpx-staphylococcal nuclease (SN) and chloramphenicol acetyltransferase (CAT) fusion proteins into HIV particles (55). Although enzymatically active, the Vpr- and Vpx-SN fusion proteins did not exert a significant antiviral effect (unpublished data). The susceptibility of SN to cleavage by the viral protease (55) may have, at least in part, accounted for the lack of measurable antiviral activity.

Several intracellular immunization strategies to inhibit HIV

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replication have been described. Of these, the expression of *trans*-dominant negative mutants (in which a genetic mutant interferes with the function performed by the parental protein) of both regulatory (Tat and Rev) and structural (Gag and Env) HIV proteins has been reported (46). Thus far, *trans*-dominant approaches have not been described for the Pol proteins. In this report, we describe studies that explore the feasibility of targeting *trans*-dominant negative mutants of Pol proteins into the virus particle by expressing a Vpx-PR-mutant (VpxPR^M) fusion protein in *trans* with HIV and SIV proviruses. We chose PR as our candidate protein to initiate these studies since the formation of PR heterodimers is known to alter enzyme function, the phenotype of PR-defective virus is relatively easy to monitor, and there are well-characterized inhibitors to the viral protease available for comparison. Our data show that VpxPR^M can be efficiently incorporated into HIV-2 and SIV particles. Virions containing the mutant fusion protein exhibit increased amounts of unprocessed Gag and Gag/Pol precursor proteins. Moreover, particle infectivity of PR^M-complemented virions is markedly reduced. These results indicate that it is possible to interfere with the function of the viral protease by *trans* complementation with a PR mutant.

MATERIALS AND METHODS

Cells and viruses. HeLa-tat (HLtat) cells constitutively express the first exon of HIV-1 tat (12) and were used in transfection assays to facilitate high levels of virus production as previously described (53). HLtat cells were maintained in Dulbecco's modified Eagle's medium supplemented with 10% fetal bovine serum. Human peripheral blood mononuclear cells (PBMC) were obtained from healthy adult volunteers by Ficoll-Hypaque density gradient centrifugation. PBMCs were stimulated with phytohemagglutinin (2 µg/ml) for 24 h, washed free of lectin, and maintained in complete medium (RPMI 1640 medium supplemented with 2 mM L-glutamine, 100 µg of gentamicin per ml, 20% fetal bovine serum, and 30 U of interleukin-2 [Boehringer Biologicals] per ml). The HIV-2_{ST} (30) proviral clone was used for the construction of recombinant expression vectors. The HIV-2_{7312A} (unpublished data) and SIV_{SM/PB1.9} (8) replication-competent proviral clones were used to produce virus by transfection into mammalian cells.

Plasmid construction. PCR methods were used to mutate the PR coding sequence at position 25, changing the conserved aspartic acid to a threonine residue. Briefly, two partially overlapping DNA fragments encompassing the entire PR coding region were amplified from HIV-2_{ST} proviral DNA by PCR in separate reactions. The 5' fragment was amplified by using primers (sense [5'-CGAGCTCGGTACCCGGGCTCAATTCTCTTTGGAAA-3'] and antisense [5'-CCCCGGTTAAACAACTTCTACTGG-3']) containing *Sst*I and *Hpa*I sites, respectively (underlined). The 3' fragment was amplified by using primers (sense [5'-CCCCGGTTAAACAGGGGCTGACGACTCAATA-3'] and antisense [5'-GCGTCGACTCATAGATTTAATGACATGCCTAA-3']) containing *Hpa*I and *Sal*I sites, respectively (underlined). The overlapping internal primers substituted a G with an A, an A with a C, and a C with an A at positions 2155, 2156, and 2157, respectively (boldface). The two PCR products were digested with appropriate restriction enzymes and ligated into the polylinker of the pTM-1 vector (38) at the *Sst*I and *Sal*I restriction enzyme sites, generating pTM-PR^M. The mutant PR coding region (PR^M) was then excised from pTM-PR^M with *Sst*I and *Sal*I and ligated together with a vpx-containing *Nco*I-*Sst*I DNA fragment (excised from the pTM-vpxSN expression vector [55]) into the *Nco*I and *Xho*I restriction sites of the pLR2P expression plasmid (55), generating pLR2P-vpxPR^M (Fig. 1A). A second VpxPR^M construct was made in order to preserve the N-terminal PR cleavage site. The 5' end of the PR reading frame and 48 bp of the upstream *trans*-frameshift (TF) sequence were amplified by PCR using primers (sense [5'-GCGGAGCTCGCTCGAGAAACAAGA GAC-3'] and antisense [5'-CCCCGGTTAAACAACTTCTACTGG-3']) containing *Sst*I and *Hpa*I sites, respectively (underlined). This DNA fragment was ligated into the *Sst*I and *Hpa*I sites of pTM-PR^M, generating pTM-TFPR^M. The TFPR^M-containing *Sst*I-*Sal*I DNA fragment was then excised from pTM-TFPR^M and ligated together with a vpx-containing (truncated by 51 bp on the 3' end) *Nco*I-*Sst*I DNA fragment into the pLR2P vector that was prepared by digestion with *Nco*I and *Xho*I, generating pLR2P-vpx_{Δ17}TFPR^M (Fig. 1B). A truncated vpx gene fragment was used to avoid possible obstruction of the PR cleavage site by the seven prolines that are positioned at the N terminus of Vpx. This truncation does not alter Vpx packaging (unpublished data). A packaging-defective VpxPR^M construct was also made by deleting 44 amino acid residues from the C terminus of Vpx by procedures similar to those described above. Briefly, a PCR-amplified *Nco*I-*Sst*I DNA fragment (containing vpx truncated by 132 bp) was ligated together with an *Sst*I-*Sal*I PR^M-containing DNA fragment into

FIG. 1. Construction of the VpxPR^M and Vpx_{Δ17}TFPR^M expression plasmids. (A) Illustration of the fusion junction of the pLR2P-vpxPR^M expression plasmid. An *Sst*I-*Sal*I DNA fragment containing the HIV-2_{ST} PR gene mutagenized at position 25 (PR^M) was ligated together with an *Nco*I-*Sst*I vpx-containing DNA fragment into the pLR2P expression vector at *Nco*I and *Xho*I restriction sites. The DNA fragment containing vpx was obtained from the pTM-vpxSN vector that had been mutagenized previously, changing the translational stop codon (TAA) to serine and substituting a valine with an arginine residue at position 112. (B) Illustration of the fusion junction of the pLR2P-vpx_{Δ17}TFPR^M expression plasmid. An *Sst*I-*Sal*I DNA fragment containing the PR^M coding region as well as 48 bp of the upstream TF sequence (TFPR^M) was ligated together with an *Nco*I-*Sst*I vpx-containing DNA fragment into the pLR2P expression vector at *Nco*I and *Xho*I restriction sites. The DNA fragment containing vpx was generated by PCR, deleting 51 bp (or 17 amino acid residues; Vpx_{Δ17}) of sequence from the C terminus. The vpx and PR coding regions are shaded.

pLR2P that had been prepared by digestion with *Nco*I and *Xho*I, generating pLR2P-vpx_{Δ44}PR^M.

Antibodies. Antibodies used in this study included the monoclonal antibody RIC7, reactive with the p27^{gag} protein of HIV-2 and SIV_{MAC} (37, 54), and the rabbit antiserum R4031, raised against the N-terminal 19 amino acid residues of the HIV-2 Vpx protein (23). The antiprotease antiserum (R667) was generated against HIV-2 PR expressed in *E. coli*. Briefly, the PR coding region, plus 48 bp 3' of PR (encoding 16 amino acids of the TF region), was cut from pTM-TFPR^M (see above) and ligated into the pGEX vector by using *Nco*I and *Sal*I restriction enzymes. This construction positioned TFPR^M in the pGEX vector for expression in *E. coli* as a fusion protein with glutathione S-transferase as described previously (39). New Zealand White female rabbits were immunized subcutaneously with 200 µg of purified TFPR^M protein emulsified in an equal volume of Freund's complete adjuvant. Rabbits were boosted at 2-week intervals with 200 µg of TFPR^M protein mixed with an equal volume of Freund's incomplete adjuvant. Serum titers were determined, and sera were analyzed for specificity by immunoblotting against purified preparations of both the immunizing protein and concentrated HIV-2_{ST} virions.

Transfections and virus purification. Transfections of proviral clones and recombinant expression plasmids were performed in monolayers of 40 to 60% confluent HLtat cells by using the calcium phosphate DNA precipitation method as recommended by the manufacturer (Stratagene). Forty-eight hours after transfection, culture supernatants were collected and clarified by low-speed centrifugation (1,000 × g, 10 min). Virions were then concentrated by ultracentrifugation (125,000 × g, 2 h) through cushions of 20% sucrose. Viral pellets were either solubilized in loading buffer for immunoblot analysis or resuspended in TNE buffer (10 mM Tris-HCl [pH 7.2], 100 mM NaCl, 1 mM EDTA) for sucrose gradient analysis.

Immunoblot analysis. Transfected HLtat cells monolayers and virus pellets were solubilized in loading buffer (62.5 mM Tris-HCl [pH 6.8], 0.2% sodium dodecyl sulfate [SDS], 5% 2-mercaptoethanol, 10% glycerol), boiled for 5 min, and separated on polyacrylamide gels containing SDS. Following electrophoresis, proteins were transferred to nitrocellulose (0.2-µm pore size; Schleicher & Schuell) by electroblotting, and incubated for 1 h at room temperature in blocking buffer (5% nonfat dry milk in phosphate-buffered saline [PBS]) and then for 2 h with the appropriate antibodies diluted in blocking buffer. The Gag monoclonal antibody was used at a dilution of 1:1,000, and the anti-Vpx and anti-PR antisera were used at dilutions of 1:500. Protein bound antibodies were probed with horseradish peroxidase-conjugated specific secondary antibodies (used at 1:2,000 dilutions), washed extensively, and developed by using the highly sensi-

FIG. 2. Expression and incorporation of the VpxPR^M and Vpx_{Δ17}^{TF}PR^M fusion proteins into HIV-2 by *trans* complementation. HIV-2_{7312A} proviral DNA (4 μg) was transfected into HLTat cells alone and cotransfected separately with pLR2P-vpxPR^M (4 μg) and pLR2P-vpx_{Δ17}^{TF}PR^M (4 μg). Forty-eight hours after transfection, extracellular virus was concentrated from culture supernatants by ultracentrifugation (125,000 × g, 2 h) through cushions of 20% sucrose. Pelleted virions were lysed in loading buffer and examined by immunoblot analysis. Replica blots were prepared and probed independently with anti-Vpx (A) and anti-PR (B) antibodies.

tive enhanced chemiluminescence detection system as recommended by the manufacturer (Amersham).

Infectivity assay. Virus infectivity was assessed by endpoint dilution assay. Forty-eight hours after transfection of HLTat cells, culture supernatants were collected, clarified by low-speed centrifugation (1,000 × g, 10 min), filtered through 1.2-μm-pore-size filters, and analyzed for p27 antigen content by enzyme-linked immunosorbent assay (ELISA; Coulter Inc.) and for RT activity by previously described methods (10). Six serial fivefold dilutions of p27 antigen-equilibrated supernatants were made in complete medium and used to infect cultures of phytohemagglutinin-stimulated PBMC. After 24 h, cells were washed and monitored for virus replication by p27 antigen ELISA every 3 to 4 days for a 21-day period.

RESULTS

Expression and incorporation of VpxPR^M into HIV-2 particles. Two different VpxPR^M expression plasmids were constructed. The pLR2P-vpxPR^M construct contained the full-length *vpx* gene fused to the 5' end of the PR coding region. To preserve the natural N-terminal PR cleavage site, pLR2P-vpx_{Δ17}^{TF}PR^M was constructed by fusing a truncated *vpx* coding region to the TF sequence, 48 bp 5' of PR (Fig. 1). Expression and virion incorporation of the VpxPR^M fusion proteins were assessed by separately cotransfecting HIV-2_{7312A} DNA with the pLR2P-vpxPR^M and pLR2P-vpx_{Δ17}^{TF}PR^M expression plasmids. The HIV-2_{7312A} clone was transfected by itself as a control. Forty-eight hours later, extracellular virions were pelleted from culture supernatants by ultracentrifugation through cushions of 20% sucrose, lysed, and then examined by immunoblot analysis (Fig. 2). Anti-Vpx antiserum detected the 15-kDa native Vpx protein in virions derived from all cultures (Fig. 2A). Strongly reactive proteins migrating at approximately 25 and 27 kDa were detected in virions derived by coexpression of HIV-2 with VpxPR^M and Vpx_{Δ17}^{TF}PR^M, respectively. These proteins migrated as expected on the basis of their calculated molecular weights. Anti-PR antiserum also detected VpxPR^M and Vpx_{Δ17}^{TF}PR^M at 25 and 27 kDa, respectively, confirming expression of the PR^M partner of the fusion protein (Fig. 2B). The HIV-2-encoded protease was detected only after prolonged exposures (data not shown), presumably because substantially less PR is naturally incorporated through the expression of the Gag/Pol compared with *trans* expression of VpxPR^M.

Both fusion proteins were found to be susceptible to cleavage by the viral protease. Cleavage products of the Vpx_{Δ17}^{TF}PR^M fusion protein were detected at approximately

FIG. 3. Immunoblot analysis of HIV-2 virions *trans* complemented with the VpxPR^M, Vpx_{Δ17}^{TF}PR^M and VpxCAT fusion proteins. HIV-2_{7312A} proviral DNA (4 μg) was transfected into HLTat cells alone and cotransfected separately with an equal concentration of pLR2P-vpxPR^M, pLR2P-vpx_{Δ17}^{TF}PR^M, pLR2P-vpx_{Δ44}PR^M, and pLR2P-vpxCAT. Forty-eight hours after transfection, extracellular virus was concentrated from culture supernatants by ultracentrifugation (125,000 × g, 2 h) through cushions of 20% sucrose. Pelleted virions (A and B) and cell monolayers (C) were lysed with loading buffer and examined by immunoblot analysis. Replica blots prepared with lysates of viral pellets were probed separately with anti-Gag (A) anti-Vpx (B) antibodies. Blots prepared using selected cell lysates were probed with anti-Vpx antiserum (C).

11 and 15 kDa (Fig. 2B). The 11-kDa protein is thought to represent PR^M after its release from the fusion protein by hydrolysis of the N-terminal PR cleavage site. Detection of the 15-kDa protein is also consistent with the release of PR^M, since it likely represents Vpx (truncated by 17 amino acids) plus the 16 amino acid residues encoded by the fused TF sequence (Vpx_{Δ17}^{TF}). The anti-PR antiserum was raised against a bacterially expressed PR that contained this portion of the TF sequence (see Materials and Methods). Cleavage products of the VpxPR^M fusion protein were also detected at approximately 17 and 20 kDa (Fig. 2). Since the anti-Vpx antiserum is specifically reactive to the N terminus, this result indicates that the VpxPR^M fusion protein must have been cleaved within PR, possibly as a result of structural alterations induced by expression as a VpxPR^M fusion protein. A different possibility is that PR is naturally susceptible to cleavage at a site near its N terminus (1). To exclude the possibility that VpxPR^M and Vpx_{Δ17}^{TF}PR^M were enzymatically active, they were transfected alone and cotransfected separately with the PM3 PR-defective provirus (25). Neither fusion protein exhibited autoproteolysis when expressed alone or the ability to cleave the p55^{gag} precursor when coexpressed with PM3 (data not shown). These results demonstrate that VpxPR^M fusion proteins can be efficiently expressed *trans* with HIV-2 provirus and incorporated into virions, even in the presence of wild-type Vpx protein. In addition, these data show the viral protease can recognize the N-terminal PR cleavage site and release the PR^M component of the Vpx_{Δ17}^{TF}PR^M fusion protein.

Virions *trans* complemented with VpxPR^M contain increased amounts of unprocessed Gag and Gag/Pol precursor proteins. Wild-type virions and those derived by coexpression of HIV-2 with VpxPR^M and Vpx_{Δ17}^{TF}PR^M were analyzed by immunoblot analysis using Gag monoclonal antibodies. Greater relative amounts (compared with p27) of unprocessed p55^{gag} precursor protein were detected in both VpxPR^M- and Vpx_{Δ17}^{TF}PR^M-containing virions (Fig. 3A). In addition, these virions contained easily detectable amounts of a 160-kDa protein, which likely represents unprocessed Gag/Pol precursor protein. Important controls used in this analysis included pLR2P-vpx_{Δ44}PR^M and pLR2P-vpxCAT. pLR2P-vpxCAT ex-

FIG. 4. Dosage response effect of VpxPR^M fusion protein on Gag and Gag/Pol processing. Different concentration of pLR2P-vpxPR^M, ranging from 1 to 8 μ g, were cotransfected with constant amounts HIV-2 proviral DNA (4 μ g) into HLTat cells. Forty-eight hours after transfection, extracellular virus was concentrated from culture supernatants by ultracentrifugation ($125,000 \times g$, 2 h) through cushions of 20% sucrose. Pelleted virions were lysed with loading buffer and examined by immunoblot analysis using anti-Gag antibodies.

presses a VpxCAT fusion protein of approximately 40 kDa that is efficiently packaged into HIV-2 virions when coexpressed in *trans* (55). This vector was included to address the possibility that the virion associated Vpx fusion protein may nonspecifically interfere with the processing of the Gag and Gag/Pol precursors. The packaging defective pLR2P-vpx Δ_{44} PR^M expression vector was included to address whether virion association of VpxPR^M was required for its inhibitory effect on Gag and Gag/Pol processing. Results of immunoblot analysis demonstrated that the Gag protein profiles of virions *trans* complemented with VpxCAT and Vpx Δ_{44} PR^M, respectively, resembled that of wild-type virions (Fig. 3A). Packaging of the Vpx Δ_{17} ^{TF}PR^M, VpxPR^M, and VpxCAT fusion proteins into virions was confirmed using anti-Vpx antiserum (Fig. 3B). As demonstrated previously (55), the VpxCAT fusion protein was detected migrating to a molecular mass of approximately 40 kDa. To confirm expression of the packaging defective Vpx Δ_{44} PR^M fusion protein, cell monolayers were lysed and examined by immunoblotting (Fig. 3C). The Vpx protein was detected in all cultures transfected with HIV-2_{7312A}. Additional proteins were detected in cotransfected cultures. As expected on the basis of molecular mass calculations, the Vpx Δ_{44} PR^M fusion protein was detected migrating at approximately 21 kDa and the Vpx Δ_{17} ^{TF}PR^M fusion protein (included as an additional control) was detected at 27 kDa.

The effect of VpxPR^M on Gag and Gag/Pol processing was assessed further by cotransfecting different concentrations of pLR2P-vpxPR^M with constant amounts of HIV-2 proviral DNA. Immunoblot analysis of extracellular virions pelleted from culture supernatants revealed a dosage-dependent effect. As increasing concentrations of pLR2P-vpxPR^M were used, greater amounts of unprocessed Gag and Gag/Pol were detected (Fig. 4). Identical results were found when pLR2P-vpx Δ_{17} ^{TF}PR^M was used for cotransfection (data not shown). Taken together, these results indicate that targeting VpxPR^M into virions interferes with the processing of Gag and Gag/Pol precursor proteins by the viral protease.

Sucrose gradient analysis of virions *trans* complemented with VpxPR^M. To confirm the association of the VpxPR^M with HIV-2 particles and to further investigate its impact on Gag and Gag/Pol processing, virions derived by cotransfection of HIV-2 provirus with pLR2P-vpxPR^M were sedimented in continuous gradients of sucrose and analyzed for protein content. Gag monoclonal antibody detected peak concentrations of virus sedimenting in fractions 6 and 7 (Fig. 5A), at a density of 1.16 to 1.17 g/ml (data not shown). Using anti-PR antibody, the VpxPR^M fusion protein was shown to cosediment with HIV-2 particles (compare Fig. 5A and B). Similar to results shown in Fig. 2, the fusion protein was found to be partially processed by

FIG. 5. Sucrose gradient analysis of HIV-2 and VpxPR^M *trans*-complemented HIV-2 virions. HIV-2_{7312A} proviral DNA was either transfected into HLTat cells alone (C) or cotransfected with an equal concentration of pLR2P-vpxPR^M (A and B). Forty-eight hours after transfection, extracellular virus was concentrated from culture supernatants by ultracentrifugation ($125,000 \times g$, 2 h) through cushions of 20% sucrose. Virions were resuspended in TNE, then layered over 20 to 60% linear gradients of sucrose, and centrifuged for 18 h at $100,000 \times g$ in a Beckman SW41 rotor. Fractions (0.7 ml) were collected from the bottom of the tubes, diluted in PBS, pelleted a second time by ultracentrifugation in a Beckman TL45 rotor ($125,000 \times g$, 1 h), quantified for p27 antigen content, and lysed in loading buffer for immunoblot analysis. Replica blots of virions derived by cotransfection of HIV-2 proviral DNA and pLR2P-vpxPR^M were prepared and probed separately with anti-Gag (A) and anti-PR (B) antibodies. Lysates of wild-type HIV-2 fractions were equilibrated for p27 antigen concentration with the corresponding *trans*-complemented virion fractions and examined by immunoblot analysis using anti-Gag antibodies (C). Fraction 1 represent the first collection from the bottom of the gradients, and fraction 14 represents the last.

the viral protease, and cleavage products containing Vpx plus N-terminal portions of PR were detected with anti-PR antibody. As a control, equal amounts of wild-type HIV-2 particles were analyzed in parallel experiments. Peak virus concentrations were detected with Gag monoclonal antibody in fractions 6 and 7 (Fig. 5C). By comparison with wild-type virus, substantially more unprocessed Gag protein (relative to p27) was detected in virions containing the VpxPR^M fusion protein. Also notable in the peak fractions of VpxPR^M-complemented virions was a protein detected at a molecular mass corresponding to that of the Gag/Pol precursor protein (Fig. 5A). This 160-kDa protein was not seen on blots prepared with wild-type virus, even after longer exposures.

Comparison between VpxPR^M and a protease active-site inhibitor. To further investigate the inhibitory effect of VpxPR^M on processing of the Gag precursor protein, VpxPR^M was compared with the potent active-site PR inhibitor L-689,502. The L-689,502 inhibitor was designed by molecular modeling of the HIV-1 protease structure but is also active against the HIV-2 and SIV PR (HIV-1 50% inhibitory concentration = 0.85 nM; HIV-2 50% inhibitory concentration = 12 nM [48]). In side-by-side experiments, HIV-2 and SIV_{PBJ} proviral DNAs were either separately transfected into HLTat cells or independently cotransfected with pLR2P-vpxPR^M. HIV-2- and SIV_{PBJ}-transfected cells were cultured in the presence of different concentrations of L-689,502, ranging from 0.1 to 2.7 μ M. The culture medium was exchanged after 24 h with fresh medium

FIG. 6. Comparison of the Gag protein content of HIV-2 and SIV virions *trans* complemented with VpxPR^M and those derived from cells cultured with the PR inhibitor L-689,502. Four micrograms of HIV-2_{7312A} (A) and SIV_{PBJ} (B) proviral DNAs were separately cotransfected into HLTat cells with 4 μ g of pLR2P-vpxPR^M. HIV-2_{7312A} and SIV_{PBJ} DNAs were also transfected alone and cultured in different concentrations of L-689,502 (ranging from 0 to 2.7 μ M). Forty-eight hours after transfection, extracellular virus was concentrated from culture supernatants by ultracentrifugation through cushions of 20% sucrose. Pelleted virions were lysed in loading buffer and examined by immunoblot analysis using anti-Gag antibodies.

containing the same concentration of L-689,502. After an additional 24-h period, virions were concentrated from the culture supernatants by ultracentrifugation and examined by immunoblot analysis. The results demonstrate a progressive diminution in processing of the Gag precursor protein that corresponded with increasing concentrations of L-689,502 (Fig. 6). At the higher concentrations, L-689,502 induced significant inhibitory effects on the processing of the Gag precursor protein for both HIV-2 and SIV, similar to that induced by the VpxPR^M fusion protein.

Virions containing VpxPR^M exhibit reduced infectivity. The results presented above indicate that VpxPR^M interferes with the function of the viral protease. To investigate the effects of VpxPR^M on virus infectivity, HIV-2 provirus and pLR2P-vpxPR^M were cotransfected into HLTat cells. As controls, HIV-2 was also transfected alone and cotransfected with the VpxCAT expression vector. Forty-eight hours later, supernatants were collected, filtered through 1.2- μ m-pore-size sterile filters, and assayed for p27 antigen content. Wild-type and *trans*-complemented supernatant viruses were then normalized for p27 antigen concentration, serial fivefold dilutions were made, and each was used to infect cultures of normal donor phytohemagglutinin-stimulated PBMC. Infected cultures were monitored for virus replication by p27 antigen ELISA every 3 to 4 days over a 21-day period. Endpoint dilution titers revealed reproducible differences between control (wild-type- and VpxCAT-complemented) and VpxPR^M-complemented HIV-2. In three independent experiments, VpxPR^M-containing virus was at least 25-fold less infectious than control viruses. These results (Table 1) indicate that HIV-2 virions containing VpxPR^M are impaired in the ability to infect PBMC.

DISCUSSION

We recently reported on the ability of HIV-1 Vpr and HIV-2 Vpx to direct the packaging of foreign proteins into HIV virions when expressed as heterologous fusion molecules (55). This report extends those findings by demonstrating that Vpx can be used to package HIV-2 PR^M protein into HIV-2 and SIV virions. Moreover, the data presented in this report show that the packaging of VpxPR^M inhibits the function of the viral protease, at least partially. Virions containing the fusion protein also contained increased amounts of incompletely processed Gag and Gag/Pol proteins. Consistent with these results, *trans*-complemented virions exhibited diminished levels

TABLE 1. Infectivity of HIV-2 particles *trans* complemented with VpxPR^M fusion protein

Inoculum ^a	Infectivity ^b		
	HIV-2	HIV-2 + VpxPR ^M	HIV-2 + VpxCAT
1	+	+	+
5	+	+	+
25	+	+	+
125	+	—	+
625	+	—	+
3,125	—	—	—

^a Reciprocal dilution of RT normalized virus used for inoculation per 2 $\times$ 10⁶ PBMC.

^b Results are representative of three independent experiments.

of both RT activity (data not shown; see references 16 and 43) and particle infectivity. Importantly, virions produced by coexpression with a packaging-defective VpxPR^M or the VpxCAT fusion protein contained normally processed Gag protein and were fully infectious compared with wild-type virions. These results confirmed that VpxPR^M is able to specifically inhibit the function of the viral protease after incorporation into virions.

For comparison, we analyzed the effects of a well-characterized active-site protease inhibitor (L-689,502) in parallel with that of the VpxPR^M fusion protein. At relatively high L-689,502 concentrations, significant alterations in Gag and Gag/Pol processing were observed, similar to those induced by VpxPR^M (Fig. 6). It is interesting that the same concentrations of L-689,502 which exert strong inhibitory effects on HIV-1 replication *in vitro* induce alterations in Gag processing that are subtle and not usually detectable by immunoblot techniques (11). Other studies have also shown that minor alteration in Gag and Gag/Pol processing correlate with profound effects on infectivity and virion morphology (21, 29, 45). Reports that indicate the proteolytic processing of Gag and Gag/Pol occurs in a highly ordered manner (29, 45) support the notion that the production of infectious virions is sensitive to accurate and complete processing by the virally encoded protease. While VpxPR^M exerts obvious changes in Gag processing, the transient expression system used in these investigations does not allow for the sensitive measurement of an antiviral activity. For this purpose, T-cell lines that constitutively express VpxPR^M will be needed, a goal beyond the scope of the present study.

Antiviral drugs inhibit hydrolytic activity by binding to the active site of the protease. The mechanism by which VpxPR^M inhibits normal Gag and Gag/Pol processing is not yet understood. We have considered three possibilities that are not necessarily mutually exclusive. One could be that the incorporation of VpxPR^M into virions provides excess PR substrate binding sites and thereby competes with native sites for proteolysis by the viral protease. A second possibility is that VpxPR^M is able to recognize PR binding sites on the Gag and Gag/Pol precursor proteins and thereby compete with the native PR for substrate binding. Our findings that wild-type Vpx-PR and Vpr-PR fusion proteins form homodimers and are able to hydrolyze the HIV Gag precursor protein support this view (unpublished data). Moreover, our data clearly show that VpxPR^M protein is incorporated into virions in significant excess amounts compared with the native viral PR (Fig. 3). Finally, it is conceivable that VpxPR^M forms an enzymatically inactivate heterodimer with the viral protease. This possibility is supported by studies which indicate mutation of the active

site does not induce structural alterations which inhibit dimerization (6), as well as those which show the formation of PR heterodimers can produce catalytically defective PR complexes (2, 9).

The feasibility of exploiting the virion targeting properties of HIV accessory proteins was first demonstrated by showing that CAT and SN could be packaged efficiently into HIV-2 and HIV-1 virions when expressed as fusion proteins with Vpx and Vpr, respectively (55). Since packaging of the fusion proteins occurred efficiently even in the presence of wild-type Vpx and Vpr, and since virion-associated Vpx/Vpr-CAT and SN fusion proteins retained enzymatic activity, this concept was of interest for its potential application toward the development of novel antiviral strategies against HIV replication and spread. Gene therapy approaches to control infectious diseases have been referred to as intracellular immunization (3). Several different intracellular immunization strategies, including expression of dominant negative mutants of viral gene products, inducible toxic proteins, ribozymes, antisense RNA, RNA decoys, and single-chain antibodies, have been investigated for HIV (46). This report illustrates a novel intracellular immunization-like strategy for the targeting of *trans*-dominant negative mutants of Pol proteins into virions and may offer certain advantages over the targeting of protein that are not of viral origin and other intracellular approaches. Unlike SN and CAT, the mature forms of the Pol protein are not normally susceptible to cleavage by the viral protease. Moreover, Pol proteins are not present in their mature form and fully functional until after virus assembly. Thus, virally encoded enzymes may be less amenable as targets by conventional *trans*-dominant approaches. In addition, the presence of the viral enzymes within virions is small; relative to Gag, only 5 to 10% of Gag/Pol molecules are assembled and packaged (32, 41). By fusion with Vpx or Vpr, the Pol proteins may be packaged in amounts similar to that of Gag. This report presents, for the first time, proof of principle that a virion-associated accessory protein can be used as a vehicle for targeting a *trans*-dominant negative mutant of the PR into virions, where it interferes with the function of the wild-type protein.

The ability to package VpxPR^M by *trans* complementation suggests that this approach may also provide a novel means for studies on the function of the Pol proteins. We have begun to explore this possibility with the HIV-1 PR and RT proteins. Using a recombinant vaccinia virus-T7 expression system, we have demonstrated that proteolytically active Vpr- and Vpx-PR fusion proteins can be expressed and packaged into HIV-1 and HIV-2 Gag virus-like particles, respectively (unpublished). However, because of significant intracellular proteolytic activity and cytotoxicity, an approach that utilizes mutants such as those which have been shown to reduce PR-mediated toxicity without causing significant changes in virus maturation and infectivity (27) will be necessary. Using the *trans*-complementation assay system described in this report, we have also found that Vpr-RT fusion proteins can be incorporated into HIV-1 virions and, importantly, can rescue the defect in infectivity of RT mutant proviruses (unpublished data). Moreover, HIV-1 integrase mutant proviruses appear to be rescuable (at least partially) by providing the wild-type integrase in *trans* as a Vpr-IN fusion protein (14). The ability to package functional viral enzymes independent of Gag/Pol via virion-associated accessory proteins represents a novel and potentially powerful method that may contribute toward both the study of enzyme function and the development of gene therapy-based antiretroviral strategies.

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REFERENCES

1. Babé, L. M., S. Pichuanes, P. J. Barr, I. C. Bathurst, F. R. Masiarz, and C. S. Craik. 1990. HIV-1 protease: bacterial expression, purification and characterization, p. 71–88. *In* C. S. Craik, R. Fletterick, C. R. Matthews, and J. Wells (ed.), *Proteins and pharmaceutical engineering*. Wiley-Liss Press, New York.
2. Babé, L. M., S. Pichuanes, and C. S. Craik. 1991. Inhibition of HIV protease activity by heterodimer formation. *Biochemistry* **30**:106–111.
3. Baltimore, D. 1988. Intracellular immunization. *Nature (London)* **335**:395–396.
4. Cohen, E. A., G. G. Dehni, J. G. Sodroski, and W. A. Haseltine. 1990. Human immunodeficiency virus *vpr* product is a virion-associated regulatory protein. *J. Virol.* **64**:3097–3099.
5. Cohen, E. A., E. F. Terwilliger, Y. Jalinos, J. Proulx, J. G. Sodroski, and W. A. Haseltine. 1990. Identification of HIV-1 *vpr* product and function. *J. Acquired Immune Defic. Syndr.* **3**:11–18.
6. Darke, P. L., C. L. Dilanni, J. C. Heimbach, C.-T. Leu, V. M. Garsky, R. A. F. Dixon, and I. S. Sigal. 1989. HIV-1 proteinase: substrates, inhibitors, and subunit interactions, p. 253–258. *In* H.-G. Kräusslich, S. Oroszlan, and E. Wimmer (ed.), *Viral proteinases as targets of chemotherapy*. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, N.Y.
7. Debouck, C., J. G. Gorniak, J. E. Strickler, T. D. Meek, B. W. Metcalf, and M. Rosenberg. 1987. Human immunodeficiency virus protease expressed in *Escherichia coli* exhibits autoprocessing and specific maturation of the gag precursor. *Proc. Natl. Acad. Sci. USA* **84**:8903–8906.
8. Dewhurst, S., G. E. Embretson, D. C. Anderson, J. I. Mullins, and P. N. Fultz. 1990. Sequence analysis and acute pathogenicity of molecularly cloned SIV/SMM-PBj14. *Nature (London)* **345**:636–640.
9. Dilanni, C. L., L. J. Davis, K. Holloway, W. K. Herber, P. L. Darke, N. E. Kohl, and R. A. F. Dixon. 1990. Characterization of an active single polypeptide form of the human immunodeficiency virus type 1 protease. *J. Biol. Chem.* **265**:17348–17354.
10. Dubay, J. W., S. J. Roberts, B. Brody, and E. Hunter. 1992. Mutations in the leucine zipper of the human immunodeficiency virus type 1 transmembrane glycoprotein affect fusion and infectivity. *J. Virol.* **66**:4748–4756.
11. Emini, E. A. 1995. Personal communication.
12. Felber, B. K., C. M. Drysdale, and G. N. Pavlakis. 1990. Feedback regulation of human immunodeficiency virus type 1 expression by the Rev protein. *J. Virol.* **64**:3734–3741.
13. Fisher, A. G., E. Collalti, L. Ratner, R. C. Gallo, and F. Wong-Staal. 1985. A molecular clone of HTLV-III with biological activity. *Nature (London)* **316**:636–637.
14. Fletcher, T. M., III, X. Wu, H.-M. Liu, S. McPherson, M. Newman, B. Birchachet, N. Sharova, G. M. Shaw, J. D. Boeke, M. A. Muesing, J. C. Kappes, M. Stevenson, and B. H. Hahn. Virion associated accessory proteins: new targets for antiviral therapy. *In* M. Girard and B. Dodet (ed.), *Retroviruses of human AIDS and related animal diseases*. Dixieme Colloque des Cent Gardes, in press.
15. Gabuzda, D. H., K. Lawrence, E. Langhoff, E. Terwilliger, T. Dorfman, W. A. Haseltine, and J. Sodroski. 1992. Role of *vif* in replication of human immunodeficiency virus type 1 in CD4⁺ T lymphocytes. *J. Virol.* **66**:6489–6495.
16. Göttinger, H. G., J. G. Sodroski, and W. A. Haseltine. 1989. Role of capsid precursor processing and myristoylation in morphogenesis and infectivity of human immunodeficiency virus type 1. *Proc. Natl. Acad. Sci. USA* **86**:5781–5785.
17. Guyader, M., M. Emerman, L. Montagnier, and K. Peden. 1989. Vpx mutants of HIV-2 are infectious in established cell lines but display a severe defect in peripheral blood lymphocytes. *EMBO J.* **8**:1169–1175.
18. Henderson, L. E., R. C. Sowder, T. D. Copeland, R. E. Benveniste, and S. Oroszlan. 1988. Isolation and characterization of a novel protein (X-ORF product) from SIV and HIV-2. *Science* **241**:199–201.
19. Jacks, T., M. D. Power, F. R. Masiarz, P. A. Luciw, P. J. Barr, and H. E. Varmus. 1988. Characterization of ribosomal frameshifting in HIV-1 *gag-pol* expression. *Nature (London)* **331**:280–283.
20. Kaplan, A. H., and R. Swanstrom. 1991. Human immunodeficiency virus type 1-Gag proteins are processed in two cellular compartments. *Proc. Natl.*

- Acad. Sci. USA **88**:4528–4532.
21. Kaplan, A. H., J. A. Zack, M. Knigge, D. A. Paul, D. J. Kempf, D. W. Norbeck, and R. Swanstrom. 1993. Partial inhibition of human immunodeficiency virus type 1 protease results in aberrant virus assembly and the formation of noninfectious particles. *J. Virol.* **67**:4050–4055.
 22. Kappes, J. C., J. A. Conway, S.-W. Lee, G. M. Shaw, and B. H. Hahn. 1991. Human immunodeficiency virus type 2 vpx protein augments viral infectivity. *Virology* **184**:197–209.
 23. Kappes, J. C., C. D. Morrow, S.-W. Lee, B. A. Jameson, S. B. H. Kent, L. E. Hood, G. M. Shaw, and B. H. Hahn. 1988. Identification of a novel retroviral gene unique to human immunodeficiency virus type 2 and simian immunodeficiency virus SIV_{MAC}. *J. Virol.* **62**:3501–3505.
 24. Kappes, J. C., J. S. Parkin, J. A. Conway, J. Kim, C. G. Brouillette, G. M. Shaw, and B. H. Hahn. 1993. Intracellular transport and virion incorporation of vpx requires interaction with other virus type-specific components. *Virology* **193**:222–233.
 25. Kohl, N. E., E. A. Emini, W. A. Schief, L. J. Davis, J. C. Heimbach, R. A. F. Dixon, E. M. Scolnick, and I. S. Sigal. 1988. Active human immunodeficiency virus protease is required for viral infectivity. *Proc. Natl. Acad. Sci. USA* **85**:4686–4690.
 26. Kondo, E., F. Mammano, E. A. Cohen, and H. G. Göttinger. 1995. The p6^{gag} domain of human immunodeficiency virus type 1 is sufficient for the incorporation of Vpr into heterologous viral particles. *J. Virol.* **69**:2759–2764.
 27. Konvalinka, J., M. A. Litterst, R. Welker, H. Kottler, F. Rippmann, A.-M. Heuser, and H.-G. Kräusslich. 1995. An active-site mutation in the human immunodeficiency virus type 1 proteinase (PR) causes reduced PR activity and loss of PR-mediated cytotoxicity without apparent effect on virus maturation and infectivity. *J. Virol.* **69**:7180–7186.
 28. Kramer, R. A., M. D. Schaber, A. M. Skalka, K. Ganguly, F. Wong-Staal, and E. P. Reddy. 1986. HTLV-III gag protein is processed in yeast cells by the virus pol-protease. *Science* **231**:1580–1584.
 29. Kräusslich, H.-G., M. Facke, A.-M. Heuser, J. Konvalinka, and H. Zentgraf. 1995. The spacer peptide between human immunodeficiency virus capsid and nucleocapsid proteins is essential for ordered assembly and viral infectivity. *J. Virol.* **69**:3407–3419.
 30. Kumar, P., H. Hui, J. C. Kappes, B. S. Haggarty, J. A. Hoxie, S. K. Arya, G. M. Shaw, and B. H. Hahn. 1990. Molecular characterization of an attenuated human immunodeficiency virus type 2 isolate. *J. Virol.* **64**:890–891.
 31. Lavallée, C., X. J. Yao, A. Ladha, H. Göttinger, W. A. Haseltine, and E. A. Cohen. 1994. Requirement of the Pr55gag precursor for incorporation of the Vpr product into human immunodeficiency virus type 1 viral particles. *J. Virol.* **68**:1926–1934.
 32. Layne, S. P., M. J. Merges, M. Dembo, J. L. Spuge, T. Shioda, T. Kitamura, and A. Adachi. 1992. Factors underlying spontaneous inactivation and susceptibility to neutralization of human immunodeficiency virus. *Virology* **189**:695–714.
 33. Liu, H., X. Wu, M. Newman, G. M. Shaw, B. H. Hahn, and J. C. Kappes. 1995. The Vif protein of human and simian immunodeficiency viruses is packaged into virions and associates with viral core structures. *J. Virol.* **69**:7630–7638.
 34. Loeb, D. D., R. Swanstrom, L. Everitt, M. Manchester, S. E. Stamper, and C. A. Hutchison III. 1989. Complete mutagenesis of the HIV-1 protease. *Nature (London)* **340**:397–400.
 35. Lu, Y.-L., P. Spearman, and L. Ratner. 1993. Human immunodeficiency virus type 1 viral protein R localization in infected cells and virions. *J. Virol.* **67**:6542–6550.
 36. Meek, T. D., D. M. Lambert, G. B. Dreyer, T. J. Carr, T. A. Tomaszek, Jr., M. L. Moore, J. E. Strickler, C. Debouck, L. J. Hyland, T. J. Matthews, B. W. Metcalf, and S. R. Petteway. 1990. Inhibition of HIV-1 protease in infected T-lymphocytes in synthetic peptide analogues. *Nature (London)* **343**:90–92.
 37. Minassian, A. A., V. S. Kalyanaraman, R. C. Gallo, and M. Popovic. 1988. Monoclonal antibodies against human immunodeficiency virus (HIV) type 2 core proteins: cross-reactivity with HIV type 1 and simian immunodeficiency virus. *Proc. Natl. Acad. Sci. USA* **85**:6939–6943.
 38. Moss, B., O. Elroy-Stein, T. Mizukami, W. A. Alexander, and T. R. Fuerst. 1990. New mammalian expression vectors. *Nature (London)* **348**:91–92.
 39. Newman, M. A., S. A. McPherson, T. M. Fletcher III, J. C. Kappes, and B. H. Hahn. 1995. Polyclonal rabbit antisera that detect the vpr protein of SIV_{MAC} and SIV_{SM} on immunoblots of purified virions. *AIDS Res. Hum. Retroviruses* **11**:405–408.
 40. Ogawa, K., R. Shibata, T. Kiyomasu, I. Higuchi, Y. Kishida, A. Ishimoto, and A. Adachi. 1989. Mutational analysis of the human immunodeficiency virus vpr open reading frame. *J. Virol.* **63**:4110–4114.
 41. Panet, A. D., D. Baltimore, and T. Hanafusa. 1975. Quantitation of avian RNA tumor virus reverse transcriptase by radioimmunoassay. *J. Virol.* **16**:146–152.
 42. Paxton, W., R. I. Connor, and N. R. Landau. 1993. Incorporation of Vpr into human immunodeficiency virus type 1 virions: requirement for the p6 region of gag and mutational analysis. *J. Virol.* **67**:7229–7237.
 43. Peng, C., N. T. Chang, and T. W. Chang. 1991. Identification and characterization of human immunodeficiency virus type 1 gag-pol fusion protein in transfected mammalian cells. *J. Virol.* **65**:2751–2756.
 44. Peng, C., B. K. Ho, T. W. Chang, and N. T. Chang. 1989. Role of human immunodeficiency virus type 1 specific protease in core maturation and viral infectivity. *J. Virol.* **63**:2550–2556.
 45. Pettit, S. C., M. D. Moody, R. S. Webbie, A. H. Kaplan, P. V. Nantermet, C. A. Klein, and R. Swanstrom. 1994. The p2 domain of human immunodeficiency virus type 1 gag regulates sequential proteolytic processing and is required to produce fully infectious virions. *J. Virol.* **68**:8017–8027.
 46. Pomerantz, R. J., and D. Trono. 1995. Genetic therapies for HIV infections: promise for the future. *AIDS* **9**:985–993.
 47. Sakai, K., X. Ma, I. Gordienko, and D. Volsky. 1991. Recombinational analysis of a natural noncytopathic human immunodeficiency virus type 1 (HIV-1) isolate: role of the vif gene in HIV-1 infection kinetics and cytopathicity. *J. Virol.* **65**:5765–5773.
 48. Sardana, V. V., A. J. Schlabach, P. Graham, B. L. Bush, J. H. Condra, J. C. Culbertson, L. Gotlib, D. J. Graham, N. E. Kohl, R. L. LaFemina, C. L. Schneider, B. S. Wolanski, J. A. Wolfgang, and E. A. Emini. 1994. Human immunodeficiency virus type 1 protease inhibitors: evaluation of resistance engendered by amino acid substitutions in the enzyme's substrate binding site. *Biochemistry* **33**:2004–2010.
 49. Sharp, P. M., D. L. Robertson, F. Gao, and B. H. Hahn. 1994. Origins and diversity of human immunodeficiency viruses. *AIDS* **8**:S27–S42.
 50. Sodroski, J., W. C. Goh, C. Rosen, A. Tartar, D. Portetelle, A. Burny, and W. Haseltine. 1986. Replicative and cytopathic potential of HTLV-III/LAV with *src* gene deletions. *Science* **231**:1549–1551.
 51. Strebel, K., D. Daugherty, K. Clouse, D. Cohen, T. Folks, and M. A. Martin. 1987. The HIV “A” (sor) gene product is essential for virus infectivity. *Nature (London)* **328**:728–730.
 52. Subbramanian, R. A., and E. A. Cohen. 1994. Molecular biology of the human immunodeficiency virus accessory proteins. *J. Virol.* **68**:6831–6835.
 53. Wu, X., S. Chen, H. Iwata, R. Compans, and P. Roy. 1992. Multiple glycoproteins synthesized by the smallest RNA segment (S10) of bluetongue virus. *J. Virol.* **66**:7104–7112.
 54. Wu, X., J. A. Conway, J. Kim, and J. C. Kappes. 1994. Localization of the Vpx packaging signal within the C terminus of the human immunodeficiency virus type 2 Gag precursor protein. *J. Virol.* **68**:6161–6169.
 55. Wu, X., H.-M. Liu, H. Xiao, J. Kim, P. Seshiaiah, G. Natsoulis, J. D. Boeke, B. H. Hahn, and J. C. Kappes. 1995. Targeting foreign proteins to human immunodeficiency virus particles via fusion with Vpr and Vpx. *J. Virol.* **69**:3389–3398.
 56. Yu, X.-F., M. Matsuda, M. Essex, and T.-H. Lee. 1990. Open reading frame vpr of simian immunodeficiency virus encodes a virion-associated protein. *J. Virol.* **64**:5688–5693.
 57. Yuan, X., Z. Matsuda, M. Matsuda, M. Essex, and T.-H. Lee. 1990. Human immunodeficiency virus vpr gene encodes a virion associated protein. *AIDS Res. Hum. Retroviruses* **6**:1265–1271.